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Light modulated toxicity of isoproturon toward natural stream periphyton photosynthesis: A comparison between constant and dynamic light conditions

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ABSTRACT

This study tested if a variation in light intensity, in comparison to constant light required in well-designed toxicity test, could have measurable consequences on the sensitivity of phototrophic biofilms (periphyton) to isoproturon. Two independent experiments were carried out to investigate the combined effects of light and isoproturon on the photochemical behavior of intact natural biofilms by measurements of chlorophyll fluorescence and pigment composition. Experiment 1 consisted of exposing biofilms to series of isoproturon concentrations (0–2 mg L^{-1}) for 7 h under constant light at different irradiance levels (25–300 μmol m^{-2} s^{-1}). In experiment 2, biofilms were exposed using more environmentally realistic conditions to three selected concentrations of isoproturon (2, 6 and 20 μg L^{-1}) during a 7-h-simulated daily light cycle. Our results demonstrated that light, considered here as a direct physical stressor, slightly modulated the acute toxicity of isoproturon on these diatom dominated communities. This was attributed to the fact that these two factors act specifically on the photosynthetic activity. Furthermore, it was shown that a dynamic light regime increased periphyton sensitivity to isoproturon by challenging its photoprotective mechanisms such as the xanthophyll cycle, therefore implying that traditional ecotoxicological bioassays lead to underestimate the effect of isoproturon.

1. Introduction

The use of natural freshwater for agricultural, industrial, recreational or domestic purposes has been leading to an increasing pollution of surface and ground waters with a large array of organic and inorganic micropollutants (Schwarzenbach et al., 2006). In the context of the European Water Framework Directive (WFD, 2000/60/EC) implementation, a sustained management of the aquatic ecosystems requires a reliable river toxicity assessment by developing analytical and modeling tools to probe not only the distribution and bioavailability but also the biological effects of these pollutants. In the case of herbicides, several monitoring campaigns have already consistently determined their occurrence in streams, rivers and lakes (Chèvre et al., 2008; Hoagland et al., 1996; Solomon et al., 1996). Up to now, in situ prediction of the ecological effects of toxicants on primary producers and therefore on aquatic ecosystems has been mostly based on laboratory investigations, resulting in comprehensive ecotoxicological data based on single-species tests, mainly with unicellular planktonic microalgae cultivated in standardized conditions (Nyholm and Källqvist, 1989). However these studies, which report a wide variation between species in their sensitivity to the same chemicals (Hoagland et al., 1996), lack environmental realism because they do not take into account the multiplicity of biotic (inter-specific competition, grazing, etc.) and abiotic (temperature, light, etc.) environmental factors which are potentially involved. For instance, some authors have demonstrated that the complexity of inter-specific relationships (Seguin et al., 2001; Leboulanger et al., 2001; Bérard et al., 2003; Schmitt-Jansen and Altenburger, 2007) the grazing pressure (Munoz et al., 2001) or the trophic status (Wendt-Rasch et al., 2004) may directly influence the global response of microalgae to herbicides at the community level. Other studies have pointed out that this response can depend on physiological adaptations of microalgae to environmental selective pressures such as light (e.g. Guasch and Sabater, 1998; Bérard and Benninghoff, 2001) or pollution (e.g. Bérard and Benninghoff, 2001; Dorigo and Leboulanger, 2001; Dorigo et al., 2004; Seguin et al., 2002; Bérard et al., 2003) histories. Also, the

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pattern of herbicide exposure including the possible interaction between toxicants in complex mixtures (Altenburger et al., 2004; Knauert et al., 2008) and the intensity and timing of the exposure (Tlili et al., 2008; Vallotton et al., 2008, 2009) cannot be discarded. On the other hand, the physicochemical conditions of water (i.e. dissolved organic carbon, oxygenation, etc.) can act indirectly by influencing the bioavailability (Nikkila et al., 2001; Knauer et al., 2007) and persistence (Graham et al., 1999) of contaminants. That is why experiments which better mimic field conditions have to be developed (Rohr et al., 2006), enabling us to improve accuracy in the extrapolations from laboratory bioassays to responses in natural systems at the community level (Cairns, 1983). Studies including phototrophic biofilms (also known as periphyton) are particularly recommended because they are major contributors to carbon fixation and nutrient cycling in most of the fluvial systems and are also key targets for herbicide contamination because of their ecophysiological similarities with terrestrial plants (Sabater et al., 2007; Wetzel, 2005). Since the work of Blanck and Wängberg (1988), there has been an increasing literature reporting the development of high-throughput methods allowing the assessment of structural (i.e. species succession) and functional (i.e. photosynthesis, etc.) changes in periphytic algae pertubated by toxicants (for review, see Sabater et al., 2007). Some of these studies focused on environmental factors that could modulate the effect of herbicides (Sabater et al., 2007). Among these factors, light plays a potential major role because it is a prerequisite for photosynthetic processes and is highly variable in situ on temporal and spatial scales (Hill, 1996). Some long term studies have already examined the relationship between light history and toxicity of atrazine to periphyton communities collected at different seasons and different stream sites differing in light regime due to riparian vegetation (i.e. opened and shaded sites). These authors demonstrated that higher sensitivity of periphyton to this herbicide was linked to higher light conditions prevailing during the colonization period (Guasch et al., 1997; Guasch and Sabater, 1998; Guasch et al., 1998, 2003). In contrast to these long term studies, the existing literature investigating the interactive short-term effects of light and pollutants on algae is scarce (Cho et al., 2008; Cleuvers et al., 2002; Gavis et al., 1981; Millie et al., 1992; O'Neal and Lembi, 1983; Petersen and Kusk, 2000; Wängberg and Blanck, 1988). Only one study has described joint effects of these two factors on photosynthesis and growth inhibition, but only by focusing on constant light levels (Millie et al., 1992). It is known nevertheless that light fluctuates from limiting to excessive level on diurnal and even shorter timescale due to the occurrence of clouds or movements of the streamside vegetation (Hill, 1996). Light fluctuations could have measurable consequences on the physiological responses of algae, formally known as photoacclimation (Falkowski and Laroche, 1991), which were consistently described in a previous work for natural stream periphytic communities in field conditions (Laviale et al., 2009). To our knowledge, no one has yet tried to find out the potential effect of the diurnal variability of light encountered by algae in the field on herbicide toxicity.

In this context, the aim of this study was to estimate the effect of isoproturon, a widespread photosystem II (PSII) inhibitor listed as a priority substance in the WFD, on the photosynthesis of periphyton exposed to different light conditions. An experimental setup of increasing complexity was devoted to study the combined effects of light and isoproturon on the photochemical behavior of natural biofilms by measurements of chlorophyll fluorescence and pigment composition. The algae were not only exposed to different intensities of constant light, but a daily cycle was simulated as close as possible to in situ conditions to test whether an additional physiological stress for the algae induced by dynamically changing light intensities could have measurable consequences to their sensitivity toward isoproturon.

Table 1
List of endpoints studied and nominal isoproturon concentrations ($\mu g\,L^{-1}$) tested during experiments 1 and 2 (i.e. in constant and dynamic light, respectively).

	Nominal isoproturon concentrations ($\mu g\,L^{-1}$) tested
Experiment no. 1: constant light	
F_v/F_m	0–2–20–200–2000
Φ_{PSII}	0–2–6–12–20–60–120–200–2000
NPQ	0–2–20–200–2000
Experiment no. 2: dynamic light	
F_v/F_m	
Φ_{PSII}	
NPQ	0–2–6–20
Pigments	

2. Materials and methods

2.1. Biofilms and water collection

The study area is located in the end section of the Stream Sensée, upstream near the town of Douai (Nord-Pas de Calais, France; 50°19′32″N, 3°4′6″E). This is an eutrophic site (see Laviale et al., 2009 for a detailed description) which is known to be slightly influenced by agricultural activities: the highest values for the most abundant herbicides (isoproturon, atrazine and diuron) detected monthly during the previous 12 months before biofilms collection were lower than $0.2\,\mu g\,L^{-1}$ (data from Agence de l'Eau Artois-Picardie). Natural stream phototrophic biofilms were collected twice a week from June to July by means of glass slides (76 mm × 26 mm) that were placed in racks equipped with floats. The racks were immersed vertically and parallel to the water flow, 15 cm below the surface. After 2–3 weeks of colonization, the glass substrata were transported to the laboratory within 1 h in coolboxes filled with site water and transferred in dark climate (20 °C) chamber, until the exposure experiments were initiated.

2.2. Experimental design

Two independent experiments were carried out. Experiment 1 consisted of exposing biofilms to series of nominal isoproturon concentrations between 0 and $2\,mg\,L^{-1}$ (for details see Table 1) for 7 h under constant light at different irradiance levels (25, 50, 100, 200 and 300 $\mu mol\,m^{-2}\,s^{-1}$) which were chosen following standard test protocols (Nyholm and Källqvist, 1989). Chlorophyll fluorescence was measured at the beginning and then after 1, 3 and 7 h of isoproturon exposure. In experiment 2, biofilms were exposed to 2, 6 and $20\,\mu g\,L^{-1}$ of isoproturon (nominal concentrations) over a daily light cycle which was simulated by progressive fluctuation of irradiance over 7 h. Preliminary in situ measurements of the light intensity have been made in order to determine the relevant range of irradiance to be used: 0–1000 $\mu mol\,m^{-2}\,s^{-1}$ with 20 successive steps of 15 or 30 min (Fig. 1). Due to methodological reasons, the chosen light period (7 h) was significantly shorter than in the field (around 10 h) but it was the same in experiments 1 and 2. Several glass slides were collected for fluorescence measurements and pigment analysis at simulated dawn, mid-morning, zenith, mid-afternoon and sunset (5, 350, 1000, 350 and 5 $\mu mol\,m^{-2}\,s^{-1}$, respectively). At the end of the simulated daily light cycle, all the communities were gently rinsed and transferred in uncontaminated filtered stream water for 12 h in complete darkness and a last measurement of the fluorescence signals was performed.

2.3. Incubation conditions

First, the bottoms of the slides were cleaned of any algae. Each slide was incubated horizontally in a polycarbonate vessel (VWR, Fontenay sous Bois, France) containing 60 mL of filtered

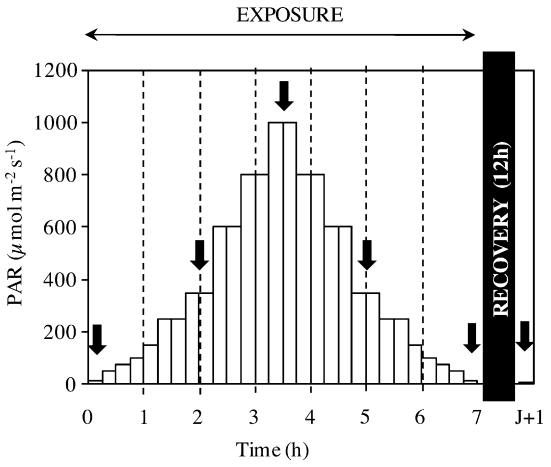

Fig. 1. Design used in experiment 2: the biofilms were exposed to 2, 6 and 20 μg L^{-1} of isoproturon over a daily light cycle which was simulated by progressive fluctuation of irradiance (0–1000 μmol m^{-2} s^{-1}) over 7 h (each open bar represents a 15-min step). Fluorescence measurements and pigment analysis were made (black arrows) at simulated dawn, mid-morning, zenith, mid-afternoon and sunset (5, 350, 1000, 350 and 5 μmol m^{-2} s^{-1}, respectively). At the end of the simulated daily light cycle, the biofilms were transferred in uncontaminated filtered stream water for 12 h in complete darkness (recovery period) and a last measurement of the fluorescence signals was performed at low light (5 μmol m^{-2} s^{-1}).

stream water (Whatman GF/F, VWR, Fontenay sous Bois, France). Each experiment was initiated within 1 day after collection. Photosynthetic activity and pigment content were monitored in a concentration series of isoproturon ([3-(4-isopropylphenyl)-1,1-dimethylurea], 99% purity,) purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). The herbicide was first diluted in acetone, and then the final nominal concentrations were obtained by diluting this stock solution with filtered stream water. Preliminary experiments were carried out with samples containing the same amount of acetone as the treatments (up to 0.1% acetone of the total volume) and indicated that the solvent had no significant effect on the photosynthetic activity. In the chosen experimental conditions, isoproturon is assumed to be quite persistent as it has been demonstrated to hydrolyze slowly (half-lives ranging from 540 to 1560 days) and to undergo photochemical degradation but mostly under UV radiations (72–88 days) (data from WHO, 2003). The incident photosynthetically active radiations (PAR) were measured at the water surface using a SA-190 2π quantum sensor connected to a Li-1400 data-logger (Li-Cor, Lincoln, USA). In experiment 1, light was provided by fluorescent tubes (36 W, Grolux, Sylvania, Genevilliers, France). In experiment 2, the biofilms were exposed to various PAR levels by changing the distance between the water surface and the actinic light source the spectrum of which simulated the visible natural sunlight (Tungsten lamp 800 W, Joker-Bug, K5600 Lighting, Bouafle, France). In both experiments, the contamination was initiated at the beginning of the light period, just after the first measurements (fluorescence and/or pigments) and water was then continuously agitated and replaced every 30 min by fresh stream water to limit nutrients depletion, to control any temperature (fixed to 20 °C) elevation due to PAR level and to prevent any variation of isoproturon concentration during the 7-h exposure.

2.4. Chlorophyll fluorescence parameters

The fluorescence signals were measured with a portable pulse-amplitude-modulated fluorometer (PAM 2100; Walz, Effeltrich, Germany; Schreiber et al., 1986). For each parameter, the measurements were made on the same spots over the entire incubation. The effective quantum yield of PSII (Φ_{PSII}) was evaluated on several slides under ambient light by means of a home-made support carrying an axis where it was possible to insert and block the tip of the optic fiber of the fluorometer at a constant distance (2 mm) from the immersed biofilm and at a 60° angle to avoid possible shading. Φ_{PSII} was calculated according to Genty et al. (1989):

$$\Phi_{PSII} = \frac{F'_m - F_t}{F'_m} \quad (1)$$

where F_t is the steady-state level of fluorescence under ambient light and F'_m is the maximum level of fluorescence measured during a saturating white light pulse (0.8 s).

The optimal quantum yield of PSII (F_v/F_m) was measured on different slides after transferring them to complete darkness for 10 min in a home-made dark incubator which allowed us to determine the fluorescence yields underwater maintaining the optic fiber modulated light probe at a standard distance (2 mm) from the intact biofilms. First, the minimum fluorescence (F_0) was determined after a weak (5 s) far red modulated light (735 nm). Then the maximum fluorescence (F_m) was reached by exposing the biofilm to a saturating pulse (0.8 s). F_v/F_m was then calculated using the Genty et al. (1989) equation:

$$\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \quad (2)$$

Preliminary results indicated that a 10 min dark acclimation period was sufficient in order to estimate accurately F_v/F_m whatever the chosen light level in experiment 1 and also at dawn and 350 μmol photons m^{-2} s^{-1} in experiment 2. However, this period should be extended at higher irradiance. As the same period was applied regardless of the irradiance (due to methodological reasons), F_v/F_m was probably underestimated at zenith.

Finally, the non-photochemical quenching (NPQ) was calculated according to the modified equation of Bilger and Björkman (1990):

$$NPQ = \frac{F^*_m - F'_m}{F'_m} \quad (3)$$

where F^*_m is the mean maximum fluorescence yield measured on dark-adapted biofilms at the beginning of each experiment.

2.5. Pigment content

In experiment 2, three glass slides for each tested isoproturon concentration were collected regularly over the simulated daily light cycle. Each glass slide was scraped with a razor blade and the suspension was gently filtered through a Whatman GF/F glass fiber filter (VWR) which was immediately frozen in liquid nitrogen and stored at −80 °C until extraction. The filters were thawed, ground and then left overnight in dim light at +4 °C in glass tubes containing 5 mL of methanol/methylene chloride (20/1, v/v). The extracts were centrifuged and supernatants were then filtered with 0.45 μm-PTFE membranes (Millipore, St Quentin en Yvelines, France). Sample volumes of 50 μL were injected into a high-performance liquid chromatography (HPLC) system equipped with a P600 photodiode array detector (Thermo, France) and a Zorbax ODS-5μC_{18} reverse phase column (Agilent Technologies, Massy, France). The separation was carried out following a method modified from Wright et al. (1991). Pigments were identified by their retention time and absorption spectra versus standards and those given in the literature (Jeffrey et al., 1997) and were quantified using external standards supplied by DHI Water and Environment (Hoersholm, Denmark). Among the quantified pigment, the molar concentrations obtained for diadinoxanthin (DD) and diatoxanthin (DT) were used to calculate the xanthophyll cycle de-epoxidation ratio (DR) as follows:

$$DR = \frac{DT}{DD + DT} \quad (4)$$

This ratio illustrates the light-induced de-epoxydation of DD into DT (Lavaud et al., 2004; Laviale et al., 2009). This reversible photoprotective mechanism helps diatoms to respond within minutes to an increase in light by dissipating excess energy as thermal radiation, similarly to what has been described to occur in higher plants and some algae with the violaxanthin–zeaxanthin cycle (Demmig-Adams and Adams, 1992; Gévaert et al., 2002).

2.6. Statistics

Values are presented as means ± 95% confidence interval (95% CI). Parametric tests were performed using the R statistical computing environment (v 2.8.1, Ihaka and Gentleman, 1996) after checking data normality and homoscedasticity using the residuals. The evolution of the fluorescence signals over the incubation was studied using a linear mixed effects model (lme function of the nlme package: Pinheiro and Bates, 2000), enabling us to take into account the fact that the measurements were made on the same spots over the 7 h (i.e. related samples). The StatXact software (v 8.0.0, Cytel Studio Inc.) was used for exact non parametric tests and multiple comparisons were performed according to the method of Siegel and Castellan (1988).

In experiment 1, the fluorescence parameters (Φ_{PSII}, F_v/F_m and NPQ) of exposed microalgae were also expressed as % of response of the mean value obtained from the control communities at the same light level after 7 h. The relationship between the relative response (y) and the isoproturon concentrations (x) was then calculated for each light level using the following log-logistic model:

$$y = c + \left[\frac{d-c}{1+e^{b(\log(x)-\log(EC_{50}))}}\right] \quad (5)$$

where EC_{50} is the concentration producing a response a half-way between the minimum (c) and maximum (d) levels observed, and b is the relative slope at the EC_{50} level. Analyses were performed using non-linear regression with least square fitting of the drc package of R (Ritz and Streibig, 2005). The EC_{50} calculated from the model fit were compared using the SI (Selectivity Indices) function that computes relative potencies.

3. Results

3.1. Experiment 1: constant light

The periphyton communities were exposed to isoproturon (0–2 mg L^{-1}) under constant light at different irradiance levels (25–300 μmol m^{-2} s^{-1}). The fluorescence parameters were estimated after 1, 3 and 7 h of isoproturon exposure. Our results

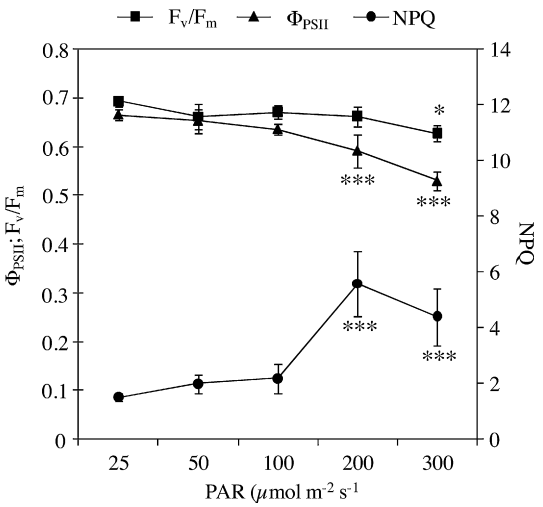

Fig. 2. Mean values (±95% CI) of F_v/F_m ((■) $n = 15$), Φ_{PSII} ((▲) $n = 30$) and NPQ ((●) $n = 30$) measured after 7 h on untreated communities incubated independently under five constant PAR levels (25, 50, 100, 200 and 300 μmol m^{-2} s^{-1}). For each fluorescence parameter, significant differences with value estimated at the lowest PAR level (25 μmol m^{-2} s^{-1}) are indicated as $^{*}p < 0.05$ or $^{***}p < 0.001$.

indicate that the effect of the herbicide was significant from 1 h and remained stable until the end of the experiment (data not shown). Fig. 2 illustrates the results obtained on control biofilms after 7 h exposure to each light level. It reveals a slight but significant (Kruskall–Wallis's test: $p < 0.05$) decrease of F_v/F_m with increasing irradiance (0.69 ± 0.01 and 0.63 ± 0.02 at 25 and 300 μmol m^{-2} s^{-1}, respectively). In the mean time, the drop of Φ_{PSII} was more pronounced (from 0.67 ± 0.01 to 0.53 ± 0.01, $p < 0.001$) whereas NPQ increased significantly ($p < 0.001$) from 1.50 ± 0.15 to 4.41 ± 0.38.

Isoproturon concentration–effect relationships were established at each light level for F_v/F_m and Φ_{PSII}, both expressed as % of response of the mean value obtained after 7 h from the control communities at the same light level (Fig. 3A and B). It appears that higher concentration of the toxicant resulted in higher inhibition of the fluorescence parameters, but the Φ_{PSII} decrease was more pronounced (100% of inhibition from 120 μg L^{-1} whatever the studied light level) than the F_v/F_m decrease (less than 60% of inhibition at 2 mg L^{-1}). This trend is summarized in Table 2 which shows that the EC_{50} ranged from 15.2 to 25.8 and 47.4 to 105.2 μg L^{-1} (or 0.07–0.13 and 0.23–0.51 μM) for Φ_{PSII} and F_v/F_m, respectively. According to the Φ_{PSII} concentration–effect relationships, the treated communities were more sensitive to isoproturon at higher light level as the EC_{50} estimated at 300 μmol m^{-2} s^{-1} was

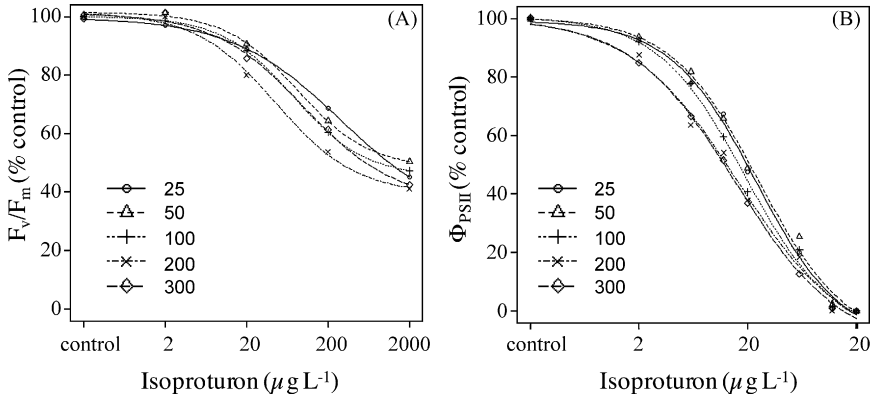

Fig. 3. Isoproturon concentration–response relationships and log-logistic modeling for communities incubated 7 h independently under 25 μmol m^{-2} s^{-1} (○), 50 μmol m^{-2} s^{-1} (△), 100 μmol m^{-2} s^{-1} (+), 200 μmol m^{-2} s^{-1} (×) or 300 μmol m^{-2} s^{-1} (◊). Mean values ($n = 6$, CI not represented for clarity) of F_v/F_m (A) and Φ_{PSII} (B) were expressed as % of control at the same light level.

Table 2
Mean values (±95% CI) of isoproturon 50% effect concentrations (EC_{50} in $\mu g\,L^{-1}$ and μM) for F_v/F_m and Φ_{PSII} estimated after 7 h on communities exposed to independent constant PAR levels ($\mu mol\,m^{-2}\,s^{-1}$). ns: the EC_{50} calculated from the model fit was not statistically different from 0 ($p > 0.05$). The same letter indicates groups which are statistically homogeneous ($p > 0.05$).

Experiment no. 1	PAR ($\mu mol\,m^{-2}\,s^{-1}$)				
	25	50	100	200	300
EC_{50} ($\mu g\,L^{-1}$)					
F_v/F_m	ns	86.6 ± 47.4[a]	76.3 ± 41.3[a]	47.4 ± 26.8[a]	105.2 ± 74.3[a]
Φ_{PSII}	24.0 ± 4.1[b]	25.8 ± 6.2[b]	20.8 ± 4.0[b,c]	16.4 ± 3.9[c]	15.2 ± 3.0[c]
EC_{50} (μM)					
F_v/F_m	ns	0.42 ± 0.23[a]	0.37 ± 0.20[a]	0.23 ± 0.13[a]	0.51 ± 0.36[a]
Φ_{PSII}	0.12 ± 0.02[b]	0.13 ± 0.03[b]	0.10 ± 0.02[b,c]	0.08 ± 0.02[c]	0.07 ± 0.02[c]

significantly lower than at 25 $\mu mol\,m^{-2}\,s^{-1}$ ($p < 0.001$). This light effect was less evident when considering F_v/F_m, as the EC_{50} were not statistically different whatever the studied light level ranging between 50 and 300 $\mu mol\,m^{-2}\,s^{-1}$ ($p \geq 0.24$). Note that the EC_{50} values are presented with respect to the actual maximal inhibition observed at each light level. Because a 100% inhibition was not observable for F_v/F_m, this implies that the corresponding EC_{50} presented may in fact describe different effect levels (around EC_{30} value). Finally, the relationship between NPQ response and isoproturon concentrations could not be described using the previous log-logistic model. Actually, both decreased and increased toxicity was observed, without a clear pattern (data not shown).

3.2. Experiment 2: simulated daily light cycle

3.2.1. Fluorescence parameters

The fluorescence parameters F_v/F_m, Φ_{PSII} and NPQ regularly measured during the simulated daily light cycle varied significantly with ambient irradiance in control and treated biofilms ($p < 0.001$). In control communities (Fig. 4), the highest F_v/F_m was observed at dawn (0.72 ± 0.01). This initial yield dropped to 0.44 ± 0.01 (or 61% of the initial mean value) when irradiance was at its maximum (1000 $\mu mol\,m^{-2}\,s^{-1}$). Then it slowly recovered, reaching 0.63 ± 0.02 at the simulated dusk, which was significantly lower than at the simulated dawn ($p < 0.05$). In comparison, the decrease of Φ_{PSII} along the morning was more pronounced, from 0.73 ± 0.01 at dawn to 0.14 ± 0.03 (19%) at zenith. Then it increased as soon as irradiance decreased and reached 0.62 ± 0.01 at the end of the simulated day. This value is significantly lower than the initial value ($p < 0.001$). NPQ showed an opposite pattern, as it gradually increased from 0.95 ± 0.01 to 10.00 ± 3.00 (around 10-fold) along the morning and then dropped to 1.67 ± 0.48 at dusk. This value was significantly higher than that at dawn ($p < 0.05$). After the 12 h dark period, F_v/F_m, Φ_{PSII} (and NPQ) mean values were higher (or lower, respectively) than those from the previous evening but recovery was not complete in comparison to initial values ($p < 0.05$).

Fig. 5 illustrates the combined effect of dynamic light and isoproturon on fluorescence parameters, including NPQ, which were reduced at increasing herbicide concentration ($p < 0.001$). The F_v/F_m values of treated communities were expressed as % of the response of the corresponding mean value obtained from the control communities at the same light level, allowing comparison of the relative effect of isoproturon between treatments at different times of measurement. According to F_v/F_m values (Fig. 5A), the effect of isoproturon was significant from mid-morning (350 $\mu mol\,m^{-2}\,s^{-1}$), even at 2 $\mu g\,L^{-1}$ (0.54 ± 0.02 or 86% of the control at the same light level, $p < 0.05$) and maximal from 6 $\mu g\,L^{-1}$ (between 56 and 70% of the control). Results obtained for 20 $\mu g\,L^{-1}$ were not significantly different from 6 $\mu g\,L^{-1}$ ($p > 0.05$) except at simulated dawn (0.37 ± 0.02 and 0.47 ± 0.02 or 59 and 75% of the control, respectively, $p < 0.01$). It appears that this inhibitory effect remained stable from mid-morning until dusk whatever the tested concentration ($p = 0.47$). The Φ_{PSII} evolution (Fig. 5B) shows that isoproturon had no consistent effect at 2 $\mu g\,L^{-1}$. The effect was maximal at 20 $\mu g\,L^{-1}$

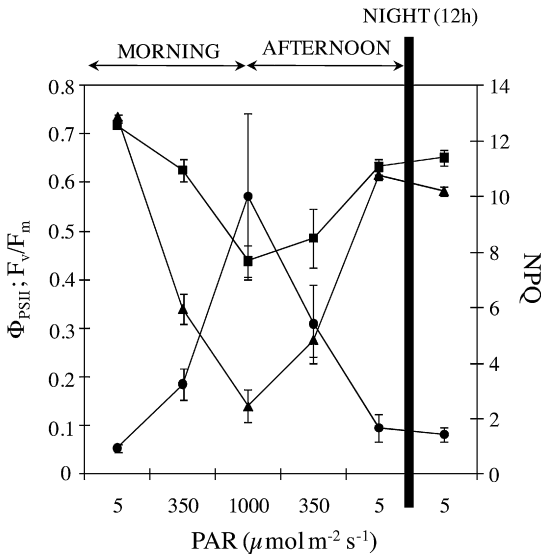

Fig. 4. Mean values (±95% CI, $n > 30$) of F_v/F_m (■), Φ_{PSII} (▲) and NPQ (●) for untreated communities exposed to PAR levels ranging from 5 to 1000 $\mu mol\,m^{-2}\,s^{-1}$ over a simulated daily cycle (7 h) and then after a 12 h dark period.

Table 3
PAR levels and mean values of F_v/F_m inhibition (%) after 7 h for communities exposed to isoproturon ($\mu g\,L^{-1}$) in constant (experiment 1) or dynamic (experiment 2) light. Values are means ± 95% CI ($n = 6$) and expressed with respect to control at the same light level after 7 h. na: data not available.

Exp no.	Instantaneous PAR ($\mu mol\,m^{-2}\,s^{-1}$)	Daily integrated PAR ($mol\,m^{-2}\,day^{-1}$)	Isoproturon ($\mu g\,L^{-1}$)		
			2	6	20
1	25	0.6	4.2 ± 3.2%	na	12.2 ± 1.8%
	50	1.3	2.9 ± 4.3%	na	13.0 ± 1.6%
	100	2.5	2.9 ± 1.7%	na	13.2 ± 1.7%
	200	5.0	2.9 ± 3.3%	na	22.1 ± 5.8%
	300	7.6	0.5 ± 1.0%	na	15.6 ± 3.1%
2	5 → 350 → 1000 → 350 → 5	10	11.9 ± 2.3%	25.0 ± 3.4%	41.1 ± 4.1%

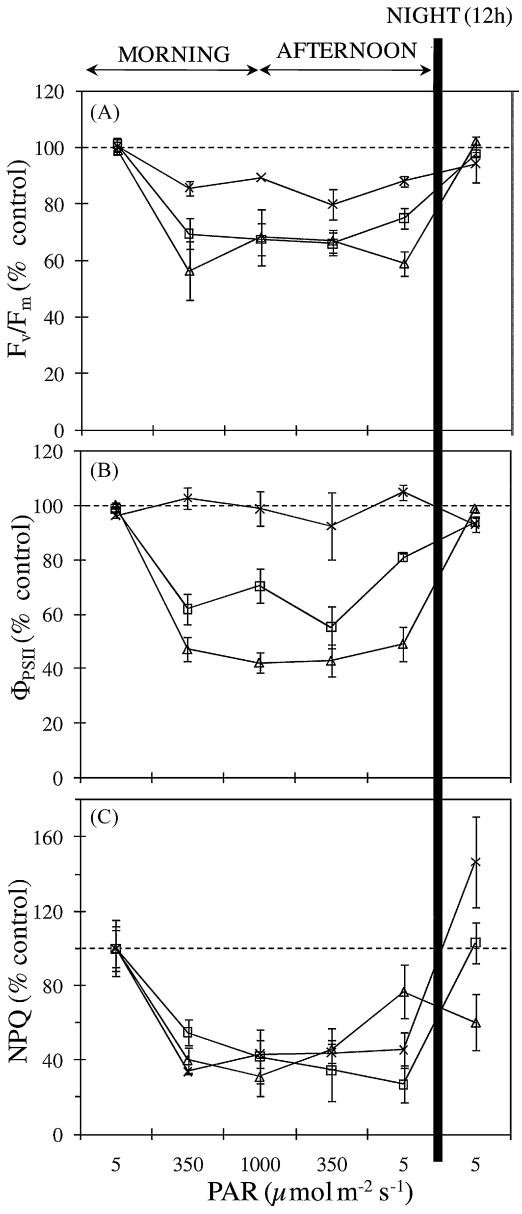

Fig. 5. Mean values (±95% CI) of F_v/F_m (A, $n = 6$), Φ_{PSII} (B, $n = 12$) and NPQ (C, $n = 6$) for communities exposed to 2 µg L^{-1} (×), 6 µg L^{-1} (□) and 20 µg L^{-1} (△) of isoproturon over the simulated daily light cycle (7 h) and then after a 12 h dark period in herbicide-free water. Values are expressed as % of control at the same light level. Dashed lines indicate the control level (100%).

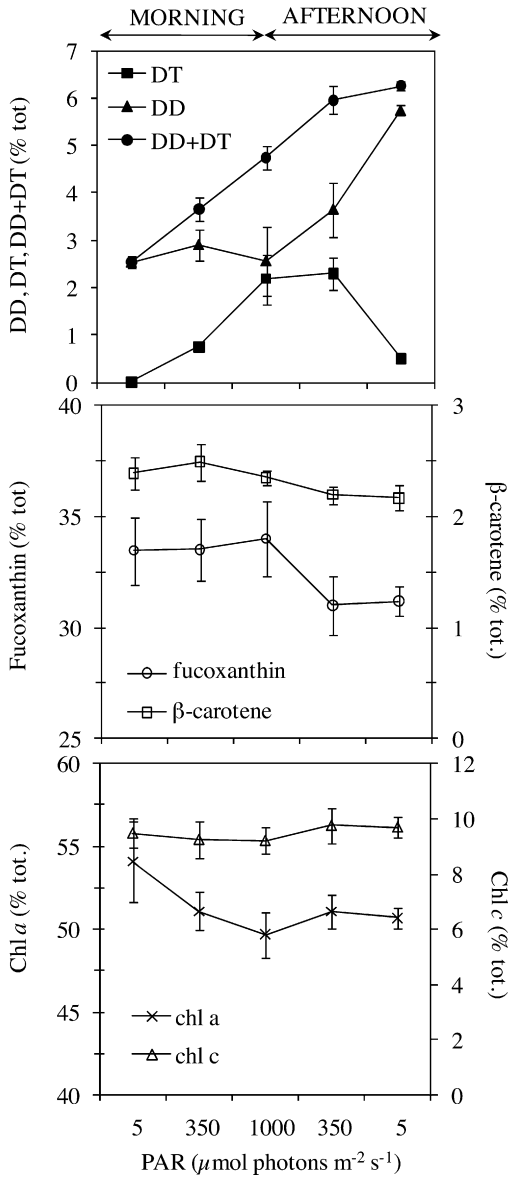

Fig. 6. Mean values (±95% CI, $n = 3$) of pigment ratio (mol per 100 mol of total pigment) for untreated communities exposed to PAR levels ranging from 5 to 1000 µmol m^{-2} s^{-1} over a simulated daily cycle (7 h). Legend: diatoxanthin (DT: ■), diadinoxanthin (DT: ▲) and diatoxanthin + diadinoxanthin (DT + DT: ●); fucoxanthin (○) and β-carotene (□); chlorophyll *a* (chl *a*: ×) and chlorophyll *c* (chl *c*: △).

with a mean value of 55% decrease along the day. Finally, it was observed that the significant decrease of NPQ ($p < 0.001$) was comparable between treatments ($p = 0.97$) with a mean 60% decrease along the day (Fig. 5C). At the end of the simulated daily light cycle, all treated biofilms were transferred into herbicide-free water and maintained in the dark. After 12 h, the recovery of F_v/F_m and Φ_{PSII} was not complete in comparison to the initial values, but comparable to the control communities ($p < 0.05$). In contrast, NPQ of the previously treated biofilms were different from the control, with the highest mean value observed for biofilms previously exposed the 2 µg L^{-1} of isoproturon, i.e. the lowest concentration ($p < 0.001$).

Finally, Table 3 offers a comparative overview of mean F_v/F_m % of inhibition calculated after the 7-h exposure to 2 and 20 µg L^{-1} of isoproturon in each experiment (i.e. at each level of constant light and at dusk of the simulated daily light cycle). It appears that biofilms were more sensitive to isoproturon for these two concentrations when a daily light cycle was carried out.

3.2.2. Pigment content

The identified pigments were chlorophyll a (chl *a*) (>50 mol per 100 mol of total pigment), fucoxanthin (>30% tot), chlorophylls *c* (chl *c*, >9% tot), DD and DT (2–6% tot) and finally β-carotene (>2% tot). This confirms preliminary microscopic observations which indicated that communities were dominated by diatoms. The evolution of the pigment content along this daily light cycle for the control biofilms (0 µg L^{-1}) is shown in Fig. 6: the proportions of fucoxanthin and β-carotene significantly decreased (Spearman's rank correlation tests: $R = -0.53$, $p < 0.01$ and $R = -0.58$, $p < 0{,}001$, respectively), whereas they increased for the pigments related to the xanthophyll cycle (DD + DT) ($R = 0.95$, $p < 0.001$), mostly due to DD ($R = 0.80$, $p < 0.001$). The proportions of chl *a* and *c* remained stable around 51.3 ± 0.8% tot ($p = 0.07$) and 9.5 ± 0.8% tot ($p = 0.88$),

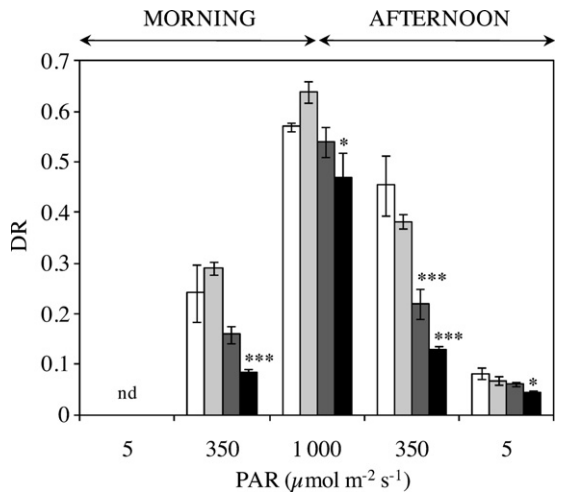

Fig. 7. Mean values (±95% CI, $n = 3$) of the de-epoxidation ratio (DR) of diadinoxanthin into diatoxanthin for controls (0 μg L^{-1}: white bar) and communities exposed to 2 μg L^{-1} (soft gray), 6 μg L^{-1} (dark gray bar) and 20 μg L^{-1} (black bar) of isoproturon over the simulated daily light cycle (7 h). At each PAR level, significant differences with control (0 μg L^{-1}) are indicated as $^{*}p < 0.05$ or $^{***}p < 0.001$. nd: diatoxanthin was not detected.

respectively. The time course of the DD de-epoxydation into DT, as illustrated by the DR values, was highly correlated to ambient light (Fig. 7, $R = 0.95$, $p < 0.001$). Note that the biofilms presented the highest DR value at zenith (0.57 ± 0.01). At 5 or 350 μmol m^{-2} s^{-1}, DR estimated in the afternoon was significantly higher than in the morning, and the same pattern was observed after isoproturon application. Moreover, isoproturon has a significant negative effect on the conversion of DD to DT ($p < 0.001$). However, this herbicide did not affect the proportions of other pigments (including DD + DT), whatever the concentrations tested or the time of measurement (data not shown, $p > 0.10$). Finally, DR and NPQ presented a similar pattern, with a significant positive correlation ($R = 0.79$, $p < 0.05$).

4. Discussion

4.1. Effect of light

This investigation tested if the variation in light intensity, in comparison to constant light required in well-designed toxicity test, could have measurable consequences on photosynthesis of natural biofilms and therefore on their response to isoproturon. The F_v/F_m values obtained at the beginning of each experiment were around 0.70, indicating that the algae were in good physiological state (Maxwell and Johnson, 2000; Laviale et al., 2009). In experiment 1, five constant light intensities (in μmol m^{-2} s^{-1}) were independently tested: 25 is far from being saturating (Hill, 1996), 50 and 100 fall into the range recommended by the international guidelines for ecotoxicological tests (Nyholm and Källqvist, 1989), 200 and 300 are comparable to light saturation levels that are regularly reported for natural stream communities (Hill, 1996).

After 7 h, untreated biofilms incubated under higher irradiance demonstrated a lower efficiency in light utilization (as reflected by the observed Φ_{PSII} decrease) which was compensated for by an increase in the conversion of energy not used in photochemistry into harmless thermal radiation (NPQ increase). At 300 μmol m^{-2} s^{-1}, it seems that this regulatory process was not enough to prevent the algae from a slight photoinhibition (F_v/F_m decrease), in comparison to values obtained at lower irradiance. In experiment 2, a daily light cycle was simulated as close as possible to in situ conditions in order to study these photoacclimation capabilities in a more environmentally realistic light environment. The time course of the fluorescence parameters reveals that the biofilms demonstrated a relative plasticity in their photobiology in response to the large variation of light encountered during the experiment (up to 1000 μmol m^{-2} s^{-1}) as previously described in field conditions (Laviale et al., 2009). This trend is reinforced by the development of the pigment content along the day which showed that an increase of the pool size of the photoprotective pigment DD was principally compensated for by a decrease of the photosynthetic fucoxanthin (Falkowski and Laroche, 1991; Lavaud et al., 2004). Furthermore, the DR was highly correlated with NPQ, which confirmed at the community level what has been described using diatom monospecific cultures: the reversible de-epoxidation of DD into DT is a larger component of NPQ in diatoms than in terrestrial plants (Lavaud et al., 2004). Finally, it was observed that the fluorescence parameters did not recover to predawn values, which could indicate that the applied light treatment was too strong. The maximum value tested in this experiment (1000 μmol m^{-2} s^{-1}) was quite close to that was obtained at zenith under clear sky between June and the beginning of July, the period at which the biofilms were collected. This is significantly lower than irradiances previously estimated in the field later in July (Laviale et al., 2009). In contrast, the sudden shortening of the light period to which the organisms were adapted before they were brought into the lab might have been an additional stressful parameter. In situ studies focusing on several photosynthetic organisms have already reported a dramatic decrease at saturating irradiance followed by a total recovery, but only after dusk (Ensminger et al., 2005; Schofield et al., 1998; Vonshak et al., 2001). For this purpose, dim light conditions (such as moon light) are known to be better suited than a complete darkness (Rmiki et al., 1996).

4.2. Effect of isoproturon

In the present study isoproturon affected the photochemical behavior of biofilms at concentrations above 2 μg L^{-1}. In experiment 1, the EC_{50} estimated for Φ_{PSII} are consistent with other studies dealing with photosynthesis of periphyton communities assessed by means of chlorophyll fluorescence method (e.g. Dorigo and Leboulanger, 2001; Schmitt-Jansen and Altenburger, 2008). This confirms that the use of Φ_{PSII} is an accurate endpoint for the assessment of acute short-term effects of phytotoxicants in constant light (Schreiber et al., 2007). In comparison, the F_v/F_m ratio was inhibited by less than 60% at the highest isoproturon concentration (2 mg L^{-1}) which exceeds the presented EC_{50} values of Φ_{PSII} inhibition by about two orders of magnitude. This is not surprising as F_v/F_m is usually used to estimate the physiological state of plants and algae (Maxwell and Johnson, 2000). Therefore its variation integrates several physiological mechanisms such as protein turnover which probably need a longer incubation time (as used in bioassays based on growth rate estimates) to develop (Horton et al., 1996). Nevertheless F_v/F_m, which is measured on dark-adapted samples, is independent of the light condition prevailing during the incubation period. Therefore it is a more suitable parameter than Φ_{PSII}, which is evaluated under ambient light, for comparing results of experiments using different light conditions (i.e. experiments 1 and 2) (see Section 4.3).

It has to be noted that the effect of isoproturon was independent of the exposure duration regardless of the light condition, which is not always the case (Gustavson et al., 2003). As a specific PSII inhibitor, this herbicide directly affected the amount of light utilization efficiency (i.e. Φ_{PSII}), thereby increasing the light energy which has to be dissipated. In addition, the herbicide resulting inhibition of electron transfer is known to lead to a relaxed proton gradient across the thylakoid membrane, which could slow down the de-epoxidation of DD to DT (Lavaud et al., 2004). This is illustrated in

experiment 2 by the correlated decrease of the DR and NPQ values in comparison to untreated biofilms. Finally, the complete recovery obtained at the community level after 12 h in herbicide-free water does not preclude exclusion of sensitive species over longer time scale (Gustavson et al., 2003; Schmitt-Jansen and Altenburger, 2008).

4.3. Joint effects of light and isoproturon

The combined effects of light and isoproturon described previously implies that the microalgae had to exert effective photoprotective mechanisms in order to limit the over-excitation of PSII illustrated here by the observed decrease of F_v/F_m values.

Our results indicated that isoproturon and light were more harmful in combination than alone, but the influence of light, although statistically significant, showed only a marginal influence. In experiment 1, the presented EC_{50} values for Φ_{PSII} did not shift by more than a factor of 2, even when the light intensity was increased by a factor of 12. In addition, the other parameters (F_v/F_m and NPQ) did not provide conclusive evidence at all. A previous study has described a light enhanced effect of simazine, a triazine herbicide, on the photosynthesis and growth of different photoacclimated (50, 130 and 230 $\mu mol\ m^{-2}\ s^{-1}$) populations of the cyanobacteria *Anabaena circinalis* (Millie et al., 1992). In contrast, another study testing several herbicides on the growth of three algae reported that the applied light treatments (2 or 10 $W\ m^{-2}$) had no general influence on the sensitivity patterns (Wängberg and Blanck, 1988).

Furthermore, the light enhanced isoproturon toxicity was slightly more pronounced in experiment 2 than in experiment 1 after the 7 h incubation. This can be attributed to the used light levels, as both daily integrated (10.0 and up to 7.6 $mol\ m^{-2}\ day^{-1}$) and maximal instantaneous (up to 1000 and 300 $\mu mol\ m^{-2}\ s^{-1}$) PAR measurements were higher in experiment 2 than in experiment 1. However, it is assumed here that the light regime (constant or dynamic) might have also influenced the biofilm response to isoproturon. Indeed, the increase of the daily integrated light intensity was around 1.3-fold whereas the inhibition of F_v/F_m was between 2.7 and 4.4 times higher in dynamic than in constant light (for 20 and 2 $\mu g\ L^{-1}$ of isoproturon, respectively). This confirms a previous study which demonstrated a significant influence of the light regime on the response of marine phytoplankton to copper, a pollutant acting on PSII photochemistry but with a more complex mode of action (Gavis et al., 1981). Finally, the modification of this molecule in potentially more toxic metabolites as a result of direct photooxidation (mostly by UV-B) has been demonstrated under natural (Knauert et al., 2008) and simulated sunlight (Dureja et al., 1991). Despite the fact that maximal light levels were higher in experiment 2 than in experiment 1, a more important accumulation of toxic metabolites in experiment 2 can be discarded here because the incubation medium was regularly replaced and the used artificial light source was simulating a natural sunlight spectrum but without UV wavelengths.

5. Conclusion

In conclusion, this study demonstrated that light, considered here as a direct physical stressor, slightly modulated the acute toxicity of isoproturon on the photosynthesis of natural phototrophic biofilms. This was attributed to the fact that these two factors act specifically on the photosynthetic activity. Furthermore, it was shown that a dynamic light regime increased periphyton sensitivity to isoproturon by challenging its photoprotective mechanisms such as the xanthophyll cycle. The three selected concentrations (2, 6 and 20 $\mu g\ L^{-1}$) are environmentally realistic, particularly during rain events after herbicidal application (Vallotton et al., 2009). Therefore these results should be taken into account for an improved risk assessment of PSII inhibitors (such as isoproturon) on primary producers in field conditions, in addition to traditional ecotoxicological tests. Our future research will focus on toxicants with different modes of action such as copper.

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